EXTRACELLULAR PECTINASE PRODUCTION BY FREE AND CALCIUM ALGINATE IMMOBILIZED CELLS OF *Enterobacter aerogenes* NBO2

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by

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LIST OF SYMBOLS AND ABBREVIATION

%	Percent
±	Plus minus
ºC	Degree Celcius
α	Alpha
β	Beta
®	Registered
µ	Micro
Δ	Delta
A	Absorbance
g	Gram
L	Litre
mg	Milligram
ml	Millilitre
mm	Millimeter
cm	Centimeter
µg	Microgram
µl	Microlitre
µmol	Micromol
M	Molar
nm	Nanometer
v/v	volume over volume
w/v	weight over volume
U	Unit

U/ml	Unit of activity per milliliter of galacturonic acid
V	Volt
g/L	Gram per litre
g/mol	Gram per mol
bp	base pair
R_f	Relative mobility
BSA	Bovine serum albumin
kDa	kilo Dalton
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
CMC	Carboxymethylcellulose
rpm	Revolutions per minute

PENGHASILAN PEKTINASE EKSTRASEL *Enterobacter aerogenes* NBO2 SECARA SEL BEBAS DAN TERSEKATGERAK DI DALAM KALSIUM ALGINAT

ABSTRAK

Sejak kebelakangan ini, enzim industri digunakan di Malaysia secara meluas disebabkan oleh kegunaannya yang pelbagai. Namun, Malaysia menghadapi kekurangan dalam penghasilan enzim industri. Justeru, perbelanjaan untuk mengimport enzim dari luar negara telah meningkatkan belanjawan kerajaan untuk sektor industri. Pektinase adalah salah satu enzim industri yang digunakan dalam industri jus dan wain. Kajian ini telah dijalankan untuk menyaring bakteria yang berpotensi menghasilkan enzim pektinase. Pencilan NBO2 telah dipilih berdasarkan keputusan penyaringan dengan penghasilan enzim yang tertinggi (1.89 ± 0.23 U/ml), dan ia telah dikenalpasti sebagai *Enterobacter aerogenes* NBO2 melalui pengecaman morfologi, biokimia dan molekular. Kajian ini bertujuan untuk meningkatkan aktiviti pektinase menerusi penambahbaikan parameter fizikal dan kimia di dalam sistem kelalang dengan menggunakan sel *E. aerogenes* NBO2 bebas dan tersekatgerak di dalam kalsium alginat. Peningkatan pektinase dengan sel bebas telah pun dijalankan dan keadaan tindak balas terbaik adalah pH awal medium 6.5, pengkulturan pada suhu 37ºC, saiz inokulum sebanyak 3.0% (i/i; $2.7x10^9$ sel/ml), kelajuan goncangan sebanyak 250 psm, 1.5% (b/i) pektin sebagai sumber karbon utama dan 0.26% (b/i) yis ekstrak sebagai sumber nitrogen. Penghasilan pektinase mencapai 18.539 U/ml selepas penambahbaikan parameter fizikal dan kimia dilakukan. Jika dibandingkan dengan profil sebelum penambahbaikan, aktiviti pektinase telah pun meningkat sebanyak 2949.18%. Seterusnya, penambahbaikan parameter fizikal dengan sel tersekatgerak *E. aerogenes* NBO2 di dalam bintilan kalsium alginat telah dilakukan. Keadaan tindak balas yang terbaik untuk sel tersekatgerak adalah, bintilan kalsium

alginat dengan kepekatan 5% (b/i), saiz inokulum sebanyak 4% (i/i; $9.3x10^8$ sel/ml), bintilan sebanyak 10 dan kelajuan goncangan sebanyak 250 psm. Penghasilan pektinase adalah sebanyak 23.478 U/ml. Jika dibandingkan profil selepas peningkatan dengan sel bebas *E. aerogenes* NBO2, peningkatan aktiviti pektinase sebanyak 26.64% telah berjaya dicapai. Pektinase tulen telah dikaji untuk berat molekulnya dengan menggunakan SDS-PAGE dan telah mendapat satu jalur tunggal. Berat molekul pektinase tulen adalah seberat 33.06 kDa. Aktiviti pektinase tulen adalah paling optimum pada suhu 40ºC dan stabil pada julat suhu 35-45ºC. Pektinase tulen paling aktif pada pH 5.5 dan stabil pada pH 5.0. Ciri-ciri pektinase tulen menunjukkan enzim tersebut boleh diaplikasikan dalam industri jus.

EXTRACELLULAR PECTINASE PRODUCTION BY FREE AND CALCIUM ALGINATE IMMOBILIZED CELLS OF *Enterobacter aerogenes* NBO2

ABSTRACT

Currently industrial enzymes are used widely in Malaysia for its vast range of importance. However the lack of locally produced industrial enzymes has increased the budget of Malaysia for industrial sector in order to import them from other countries. Pectinase is one of the industrial enzyme which is widely used in juice and wine industries. The present study was carried out to screen for the potential local bacterial pectinase producers. Isolate NBO2 was chosen based on its screening result (1.89 ± 0.23 U/ml pectinase) and was identified as *Enterobacter aerogenes* NBO2 by morphology, biochemical and molecular identification. This study was focused on the physiochemical improvements of pectinase in a flask system with free cells and immobilized cells of *E.aerogenes* entrapped in calcium alginate beads. The improved reaction conditions for pectinase production with free cells were initial medium pH of 6.5, cultivation temperature of 37ºC, inoculum size of 3.0% (v/v; $2.7x10^9$ cells/ml), agitation speed of 250 rpm, 1.5% (w/v) of pectin as a sole carbon source and 0.26% (w/v) of yeast extract as the nitrogen source. The pectinase production was 18.539 U/ml with the improved condition. There was an increment of 2949.18% compared to the activity before improvement. In order to improve the pectinase production, the physical enhancement of pectinase was carried out with immobilized cells of *E.aerogenes* NBO2 in calcium alginate. The improved reaction conditions with immobilized cells of *E.aerogenes* were calcium alginate beads with 5% (w/v) concentration, inoculums size of 4% (v/v; $9.3x10^8$ cells/ml), 10 beads and agitation speed of 250 rpm. The pectinase production was 23.478 U/ml which increased about 26.64% compared to the improved condition with free cells of *E.aerogenes* NBO2.

The purified pectinase was studied for its molecular weight with SDS-PAGE and a single band was obtained. The molecular weight of purified pectinase was 33.06 kDa. The purified pectinase was optimum at temperature 40ºC and stable at the temperature range of 35-45ºC. It was found most active at pH 5.5 and stable at pH 5.0. The characteristics of the purified pectinase showed that it is applicable in juice industries.

CHAPTER 1

INTRODUCTION

1.1 Importance of industrial enzymes in Malaysia

Enzymes present in all living cells. Thus, the enzymes can be obtainable from plant tissues, animal tissues, and microorganisms. Even though, the quantities of enzymes produced on a commercial scale from animal and plant sources are very much considerable, but for both technical and economic reasons, microbial enzymes have become increasingly important. Microbial enzymes are always available and unlimited with a higher quantity than of plant and animal sources. In addition, the microbial enzyme production can be controlled physiologically and physico-chemically which is far more convenient in commercial application of industrial enzymes. It is considered that the existence of Malaysian enzyme industry is almost none even though the import amount is quite high (Ibrahim, 2008). As for the importance of enzymes with a very large scope in the bioindustry applications, the country starts giving the priority in industrial biotechnology to develop the technologies in enzyme production.

Industrial enzymes playing an important role in producing “green” and eco-friendly processes in many industries. For an instance, a large amount of chlorine was used in the pulp and paper industry to bleach the pulp. As a result, unpleasant reaction products are formed and they are difficult to be removed from the waste stream. In order to reduce the need for chlorine bleach, enzyme xylanase which helps the separation of lignin and cellulose fibers, can be substituted (Falch, 1991).

In the production of commercial enzymes, bacterial strains are always preferred over fungal strains because of ease of fermentation process (for production) and the flexibility to implement the strain improvement techniques or any modern

technique in order to accelerate the production (Kumar and Sharma 2012). Extracellular microbial enzymes can be easily extracted, and thus making the downstream processes easier with reduced processing cost. Microbial enzyme production was performed by either submerged or solid state fermentation processes. In the last decades, a big scale production of enzyme is performed, especially via submerged fermentation system in many developed countries compared to Malaysia just because of the high production cost and capital investment in industrial applications. Malaysia used to import the enzymes to be applied in edible oil and palm oil products, oleochemical industries, detergent industries, food and beverages, manufacturing, animal feed and baking industries (Ibrahim, 2008). However, to date Malaysia is giving importance to develop industrial enzyme production technologies on its own even though there are some challenges to be faced. The rich natural resources as raw materials in Malaysia, could help to overcome the problems in developing industrial enzyme production.

1.2 Pectinase as an industrial enzyme

Pectinase is one of the important enzyme in industrial applications. Nearly 75% of the estimated sale value of industrial enzymes in l995 has been contributed by pectinases (Kashyap *et al*., 2001). Although microbial pectinase is widely used in food processing industries in other countries, it is still in its infancy in Malaysia mainly because of the high costs involved. In juice processing, enzyme is an important tool in order to improve quality and save cost. Norjana and Aziah (2011) reported that commercial microbial pectinase (Pectinex Ultra SPL (ULT) Nov Nodised Ferment Ltd., Switzerland) was successfully increased the quality and 35% (w/w) of durian juice yield with only 0.05% of enzyme concentration and 3 hours of incubation time. Similarly, commercial pectinase enzyme treatment for dragon fruit

juice also proved that there was a 0.33% (w/w) of yield increment from 0.17% (w/w) of the fresh fruit (‘Aliaa *et al*., 2010).

In beverages industry, most consumers prefer clear fruit juices. Furthermore, conventional method of pressing the fruit pulps increases the viscosity of juice which can affect the extraction and filtration processes. Thus, the amount of fruit juice extracted will be less. It is also a time and energy consuming method of fruit juice processing. Hence, the safer way of pectin removal without affecting colour and flavor is to treat the juice with pectic enzymes. Besides that, traditional method in textile industry uses toxic alkaline chemicals to remove the sizing agents. These chemicals are dangerous, causes serious environmental pollution and brings a lot of side effects on cellulose degradation. Pectinase enzymes specifically pectate lyase are the best solution to solve the problems in textile industry (Kirk *et al*., 2002).

In this study, *Enterobacter aerogenes* NBO_2 was used in the production of polygalacturonase. To the best of our knowledge, there is a very limited report on the pectinase production by this strain in submerged or solid state fermentations. Therefore, this thesis would be one of the initial studies working in this field.

1.3 Objectives of Research

1. To isolate and identify local pectinase bacterial producers.
2. To improve the cultural conditions and medium compositions for maximal pectinase production.
3. To produce pectinase by free and immobilized cells in a shake flask system.
4. To purify and characterize the pectinase produced by a bacterium.

1.4 Scope of study

The initial step of the study was the isolation of potential bacterial pectinase producers followed by screening. The highest pectinase producer was identified as *Enterobacter aerogenes* NBO2.

The enhancement of physical (pH, temperature, inoculum size and agitation speed) and chemical (carbon sources, concentration of carbon source, nitrogen source and concentration of nitrogen source) parameters were carried out to increase the pectinase production. The difference in structure of cells in various agitation speeds was viewed under SEM.

The improvement of production was further continued by using immobilized bacterial cells in a shake flask system. Calcium alginate bead as the supporting material was used for the immobilized cells. The physical parameters were enhanced to maximize the enzyme production. The structure and distribution of bacterial cells in the calcium alginate beads at 0 hour and 24 hours were viewed under SEM.

Pectinase purification was then carried out in three steps including the ultrafiltration using ultra centrifugal devices (Amicon® Ultra), partial purification and purification with gel filtration chromatography column (Sephadex G-100). The purified pectinase was performed in Sodium Dodecyl Sulphate Polyacrylamide Gel

Electrophoresis (SDS-PAGE). The molecular weight of the purified pectinase was determined by using the logarithmic graph of molecular protein, which was generated with the $\log_{10}$ of the known protein molecular and relative mobility (R_f). The purified pectinase was then characterized (temperature, thermostability, pH and pH stability).

CHAPTER 2

LITERATURE REVIEW

2.1 Pectinase enzyme

Pectinases or pectinolytic enzymes are one of the ancient enzyme that are used in homes, especially for the preparation of wines and fruit juices. However, nowadays pectinases have been used widely in commercial sectors. Pectinase function is to degrade pectic substances which can be found in the form of pectin, protopectin and pectic acids in the middle lamella and primary cell wall of higher plants (Kashyap *et al*., 2001).

Pectic substances are made up of long chains of D-galacturonic acid residues as the backbone which are linked together by a "smooth regions" of α-1,4-glycosidic linkages and carbonyl side groups with 60-90% esterified with methanol. The "hairy region" of pectic substances is a xylogalacturonan which consists of a D-xylose substituted galacturonan backbone, rhamnogalacturonan which contains rhamnose and arabinogalacturonan which contains arabinose (Venkatesh and Kumar, 2006). Rhamnose unit can be inserted into the main uronide chain and side chains like arabinan, galactan and arabinogalactan are often linked to rhamnose. Pectinase is an enzyme which helps to hydrolyze pectic substances by breaking the glycosidic bonds of galacturonic acid chains. Thus, the plant cell wall can be broken easily (Soares *et al*., 2001; Gregorio *et al*., 2002; Gummadi and Panda, 2003). Figure 2.1 shows the schematic structure of pectin.

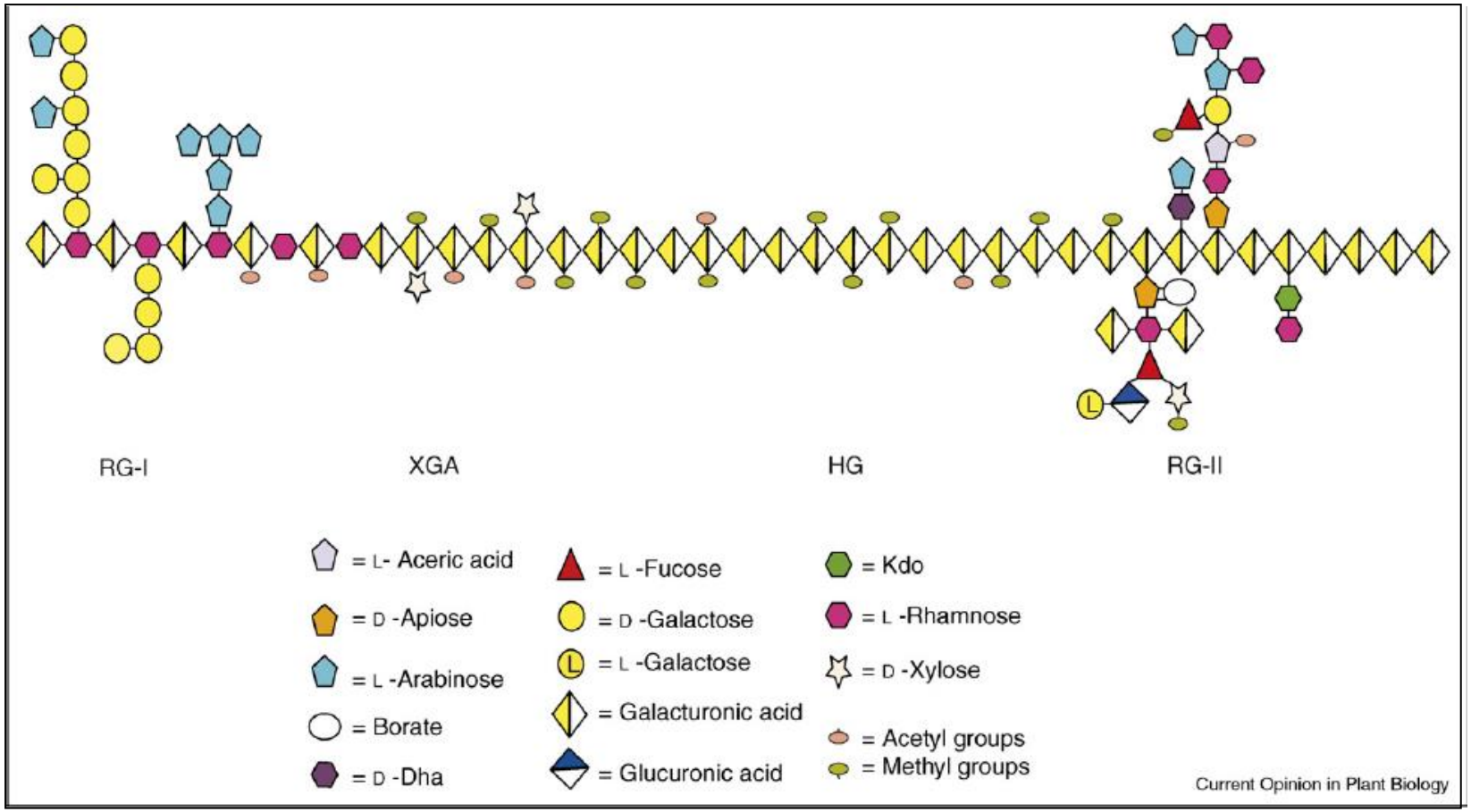

Figure 2.1: Schematic structure of pectin showing the four pectic polysaccharides homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) linked to each other (Mohnen, 2008)

2.1.1 Classification of pectinase enzyme

Since there are various forms of pectic substances present in plant cells, pectinase also exists in various forms. There are three main groups which pectinase enzyme can be classified into with the following criteria; (a) dependence on pectin, pectic acid or oligo-D-galacturonate as preferred substrate, (b) whether pectinases act by trans-elimination or hydrolysis and (c) type of cleavage whether random (endo-, liquefying of depolymerizing enzymes) or endwise (exo- or saccharifying enzymes) (Kashyap *et al*., 2001). The extensive classification of pectinolytic enzymes is given in Table 2.1 Pectinases are classified more precisely based on the mode of their action towards galacturonan part of the pectin molecule.

Pectin lyase (E.C. 4.2.2.10) is an enzyme which catalyzes highly esterified pectin and produce unsaturated methyloligogalacturonates through transelimination of glycosidic linkages. It is well known that pectin lyase is the only enzyme which acts independently in depolymerizing the pectin molecule without prior action of any other enzymes. Pectin lyase being one of the major component in commercial preparations are preferably used in fruit juice processing (Debing *et al*., 2006).

Polygalacturonases break glycosidic linkages next to free carboxyl groups by hydrolysis. They exist in two forms: endo-polygalacturonase (E.C. 3.2.1.15) which randomly breaks the pectin chain, and exo-polygalacturonase (E.C. 3.2.1.67) which releases monomers or dimers from the non-reducing end of the chain. Endo-polygalacturonase is essential in the reduction of viscosity in fruit processing products. Most of the endo-polygalacturonases are reported to be produced by fungi (Kluskens *et al*., 2005) such as the production of endo-polygalacturonase from the strains of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* by genetic

Table 2.1 An extensive classification of pectinolytic enzymes

Enzyme	E.C. no.	Modified EC systematic name	Action mechanism	Primary substrate	Product
Esterase					
1. Pectin methyl esterase	3.1.1.11		Hydrolysis	Pectin	Pectic acid + methanol
Depolymerizing enzyme					
a. Hydrolases					
1. Protopectinases			Hydrolysis	Protopectin	Pectin
2. Endopolygalacturonase	3.2.1.15	Poly-(1-4)-α-D-galactosiduronate glycanohydrolase	Hydolysis	Pectic acid	Oligogalacturonates
3. Exolpolygalacturonase	3.2.1.67	Poly-(1-4)-α-D-galactosiduronate glycanohydrolase	Hydolysis	Pectic acid	Monogalacturonates
4. Exopolygalacturonan-digalacturono hydrolase	3.1.2.82	Poly-(1-4)-α-D-galactosiduronate digalacturonohydrolase	Hydrolysis	Pectic acid	Digalacturonates
5. Oligogalacturonate hydrolase			Hydrolysis	Trigalacturonate	Monogalacturonates
6. Δ4:5 Unsaturated oligogalacturonate hydrolases			Hydrolysis	Δ4:5 $(Galacturonate)_n$	Unsaturated monogalacuronates & saturated (n-1)
7. Endopolymethyl-galacturonases			Hydrolysis	Highly esterified pectin	Oligomethylgalacturonates
8. Endopolymethyl-galacturonases			Hydrolysis	Highly esterified pectin	Oligogalacturonates
b. Lyases					
1. Endopolygalacturonase lyase	4.2.2.2	Poly-(1-4)-α-D-galactosiduronate lyase	Trans-elimination	Pectic acid	Unsaturated oligogalacturonates
2. Exopolygalacturonase lyase	4.2.2.9	Poly-(1-4)-α-D-galactosiduronate exolyase	Trans-elimination	Pectic acid	Unsaturated digalacturonates
3. Oligo-D-galactosiduronate lyase	4.2.2.6	Oligo-D-galactosiduronate lyase	Trans-elimination	Unsaturated digalacturonates	Unsaturated monogalacturonates
4. Endopolymethyl-D-galactosiduronate lyase	4.2.2.10	Poly(methyl galactosiduronate) lyase	Trans-elimination	Unsaturated poly-(methyl-D-digalacturonates)	Unsaturated methyloligogalacturonates
5. Exopolymethyl-D-galactosiduronate lyase			Trans-elimination	Unsaturated poly-(methyl-D-digalacturonates)	Unsaturated methylmonogalacturonates

Source: Jayani *et al*., (2005)

engineering (Jia and Wheals, 2000). Exo-polygalacturonase is applicable in juice extraction, wine clarification, bakery and distillery industries. Kar and Ray (2011) discovered that exo-polygalacturonase produced by *Streptomyces erumpens* MTCC 7317 increased the juice yield of vegetables and fruits for about 15-20% higher compared to the marketed pectinase, Pectinex. Similarly, exo-polygalacturonase by *Bacillus subtilis* CM5 showed 13.3% of the increment in yield of carrot juice as compared to juice extracted with Pectinex (Swain and Ray, 2010).

Pectate lyase or known as polygalacturonase lyase, splits the glycosidic linkages next to free carboxyl groups by β–elimination. They are divided into endo-pectate lyase (E.C. 4.2.2.2) and exo-pectate lyase (E.C. 4.2.2.9). Endo-pectate lyase splits the pectin chain randomly while exo-pectate lyase releases unsaturated dimers from the reducing end (Soares *et al*., 2001; Pedrolli *et al*., 2009). In cotton fabric scouring, the usage of pectate lyase enzyme showed significantly better results compared to chemical scouring which is conventional and environmentally harmful (Solbak *et al*., 2005). Studies on degumming of ramie too showed that there was a high pectate lyase activity in culture supernatant produced by *Amycolata* sp. and it was most effective in fibre separation and reduction of gum content in ramie fibre by 30% in 15 hours (Kapoor *et al*., 2001). Figure 2.2 shows the mode of action of pectinases where Figure 2.2a shows the R-H for polygalacturonase (E.C. 3.2.1.15) and CH_3 for polymethylgalacturonases, while Figure 2.2b shows the pectinesterase (E.C. 3.1.1.11) and Figure 2.2c shows R=H for pectate or polygalacturonase lyase (E.C. 4.2.2.2) and CH_3 for pectin lyase (E.C. 4.2.2.10).

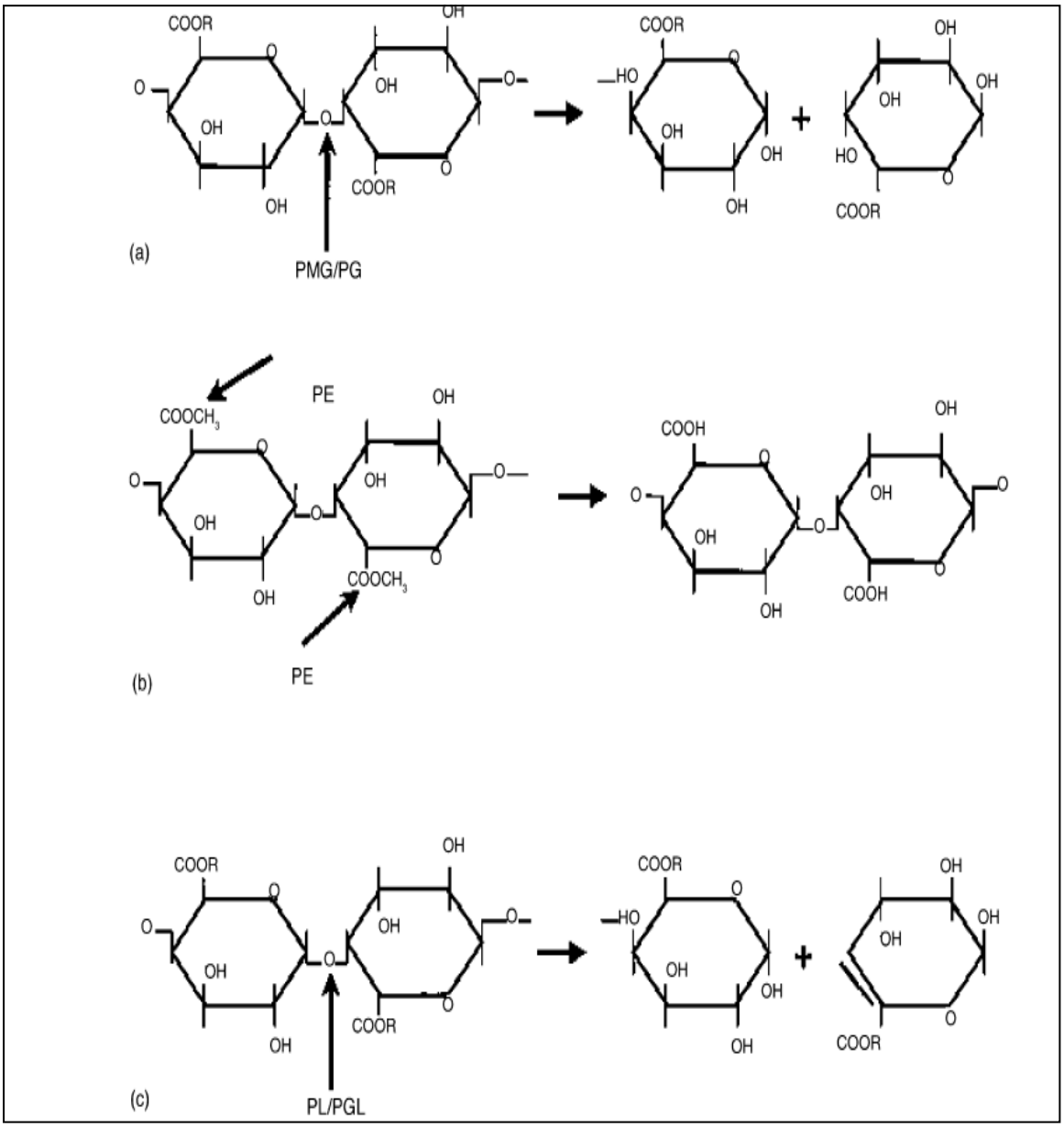

Figure 2.2: Mode of action of pectinases: (a) R=H for PG and CH_3 for PMG; (b) PE; (c) R=H for PGL and CH_3 for PL. The arrow indicates the place where the pectinase reacts with the pectic substances. PMG, polymethylgalacturonases; PG, polygalacturonase (E.C. 3.2.1.15); PE, pectinesterase (E.C. 3.1.1.11); PGL, pectate/polygalacturonase lyase (E.C. 4.2.2.2); PL, pectin lyase (E.C. 4.2.2.10) (Jayani *et al*., 2005)

Pectin methyl esterase or pectinesterase (E.C. 3.1.1.11), is a saponifying enzyme which de-esterify pectins to low methoxyl pectins and pectin depolymerases, whereby their mode of attack is by splitting the glycosidic linkages between galacturonosyl (methyl ester) residues. Its action is before polygalacturonases and pectate lyases which need non-esterified substrates. Thus, in fruit softening, first of all pectinesterase cleaves the ester bonds of pectin molecules and allows polygalacturonase to act on these bonds (Imsabai *et al*., 2002). In the study conducted by Draye and Cutsem (2008), it was reported that pectin methylesterase induces sudden increase in acidic pectin during strawberry fruit ripening.

Recently, pectin structure became advanced with "hairy regions" which is resistant to the activity of pectinases Thus, there are also several other pectinases like rhamnogalacturonase, arabinofuronosidase and xylogalacturonan hydrolases which are able to act on "hairy regions" of pectin were discovered (Venkatesh and Kumar, 2006). Arabinose is a precursor for α-fructose and L-glucose, released from the pectin polymer arabinogalacturonan which can be used as a non-calorific sweetener in low calorie foods.

2.1.2 Acidic and alkaline pectinase

Pectinase can be divided into acidic pectinase and alkaline pectinases. The efficiency of the function of the alkaline and acidic pectinases depends on the source of the pectin. Alkaline pectinases act well with fibre pectins while acidic pectinase plays a good role in food processing due to the acidic pH of fruit juices.

Alkaline pectinases are useful in textile and plant fibre processing, oil extraction, treatment of industrial wastewater containing pectinacious material, purification of plant viruses, paper making and even in coffee and tea fermentation (Li *et al*., 2005). It has been found that the pectinolytic capability of alkaline

pectinases on cotton pectin is nearly 75% higher than that of acidic pectinases (Agrawal *et al*., 2007). Alkaline pectinases can be produced by a variety of microorganisms, and it was reported that many of them are alkaliphilic *Bacillus* sp. but also some filamentous fungi and yeasts (Li *et al*., 2005; Pedrolli *et al.*, 2009). However, production of alkaline pectinases is still exploited to a limited extend as there are very few reports available on their applications. Among them, *Bacillus subtilis* WSHB04-02 was reported to produce alkaline pectinase which was used in bioscouring of cotton knitted fabrics (Wang *et al*., 2007).

Acidic pectinases on the other hand, are used largely in extraction, clarification and the removal of pectin in fruit juices, maceration of vegetables in order to produce pastes and purees and also in the wine industry. Acidic pectinases are widely produced by filamentous fungi especially *Aspergillus niger* (Pedrolli *et al*., 2009). *Aspergillus* sp. UU1 was also reported to produce acidic pectinases (Sunnotel and Nigam, 2002). Recently, most studies on pectic enzymes have been emphasized on the production and applications of acidic pectinases.

2.2 Producers of pectinase enzyme

Pectinase enzyme can be produced by plants, insects, nematodes, protozoan, bacteria, fungi and yeast (Pedrolli *et al*., 2009). Although, a large number of organisms producing pectinases have been reported, selection of potential isolates remains a tedious task especially, when physiologically potential strains are to be obtained to achieve maximum yield (Patil and Dayanand, 2005).

2.2.1 Plants

Pectin is the jelly-like matrix which cements the plant cells together. Thus, in natural processes of plants such as maturation, fruit softening and ripening, pectinase

is produced to break down the pectin molecules so that further processes can be continued. For instance, pectinase enzyme present in abscission zone of unifoliate (*Phaseolus vulgaris*) leaves for the dissolution of pectic substances and the formation of separation layers (Morre, 1968). Similarly, Yager (1960) reported that pectin methylesterase was found in the abscission zone of tobacco flowers. There are also studies conducted on the role of pectinase enzyme in fruit ripening and softening. Pectinolytic enzymes were detected in strawberries ripening (Draye and Cutsem, 2008). Softening of durians is highly correlated with the polygalacturonase activity which is sensitive to temperature compared to pectinesterase activity (Imsabai *et al*., 2002).

2.2.2 Microorganisms

As it is known earlier, the microorganism is the major source of enzymes. Pectinase enzyme is one of the most widely distributed enzymes in filamentous fungi, bacteria and yeast. Pectinolytic microorganisms can be isolated from soil, spoiled fruits, vegetables, decayed leaves, wood and water samples taken from decaying coconut husks especially in Coastal areas. They are also found in the intestinal flora of human, specifically bacteria where they consume pectin (dietary fiber) as their substrate (Bhardwaj, 2010). Fermentation with a suitable microorganism cultivated in the medium contained pectin, can induce the pectinase production (Venkatesh *et al*., 2009). The selection of microbial source for polygalacturonase production is based on a few features, such as the type of culture (solid state or submerged fermentations), the number and type of the produced pectinase, pH and thermal stability of the enzymes and genotypic characteristic of the strain (Khairnar *et al*., 2009).

2.2.2.1 Filamentous fungi

Pectinase-producing fungus *Aspergillus niger*, is used widely in industries because of its status as GRAS (generally regarded as safe) so that metabolites produced by this strain can be used confidently. This strain can produce different pectinases such as polymethylgalacturonase, polygalacturonase, and pectin esterase (Janani *et al*., 2011). Palaniyappan *et al*., (2009) has reported 6.1 U/ml of pectinase production by *Aspergillus niger* (MTCC: 281). Moreover, there is also a report on *Aspergillus niger* producing 328 U/l of polygalacturonase by using pressed apple pulp as a carbon source (Mojsov, 2010). Other species of *Aspergillus* such as *Aspergillus carneus* NRC1 produced 40 mg/ml of pectinase with an optimum temperature of 55ºC and pH of 4.0 (El-Sheekh *et al*., 2009). *Aspergilus fumigatus* which is a thermophilic fungus also can produce 1116 U/g of pectinase and 1270 U/g of polygalacturonase (Phutela *et al*., 2005). Despite, *Trichoderma* species also can produce the pectinase enzyme. However, only a few species like *Trichoderma lignorum* and *Trichoderma reesei* are recorded as the good pectinase producers (Gregorio *et al*., 2002). *Penicillium* is a world wide known fungus for secondary metabolites and extracellular enzyme production of commercial value, where newly isolated *Penicillium chrysogenum* was reported to produce the pectinase enzyme with a molecular weight of 31 kDa (Banu *et al*., 2010). *Penicillium viridicatum* RFC3 produced high amounts of polygalacturonase and pectin lyase by using agricultural wastes as carbon sources (Silva *et al*., 2002).

2.2.2.2 Bacteria

Lately, almost all the pectinolytic enzymes used in industrial applications are produced by fungi. However, only a few reports are about bacterial pectinase.

Bacteria are preferred for their character which is not being influenced by climatic and seasonal changes (Prathyusha and Suneetha, 2011). Several species of bacteria producing pectinases are *Agrobacterium tumefaciens*, *Bacteroides thetaiotamicron*, *Ralstonia solanacearum*, *Bacillus* sp., *Bacillus polymyxa*, *Erwinia* spp., *Erwinia carotovora*, and *Pseudomonas syringae* (Jayani *et al*., 2010; Gregorio *et al*., 2002). A paper was published by Bayoumi *et al*. (2008) about the production of polygalacturonase (515 U/ml) by *Bacillus firmus* –I-4071 via solid state fermentation (SSF). *Bacillus sphaericus* (MTCC 7542) can produce polygalacturonase up to 6.4 ± 0.8 µmol/ml/min (Jayani *et al*., 2010). Moreover, *Bacillus pumilus* dcsr1 can produce highly alkaline (pH 10.5) and thermostable pectinase (temperature 50ºC) by submerged fermentation (Sharma and Satyanarayana, 2006). Sittidilokratna *et al*., (2007) reported that *Bacillus* sp. strain N10 can produce high level of pectinase enzyme which soften the pulp and separated the fibers which was clearly seen under scanning electron microscope. Beside *Bacillus* sp., *Streptomyces* sp. RCK-SC was reported to produce thermostable alkaline pectinase (Kuhad *et al*., 2004). Furthermore, the pectinase of *Pseudomonas fluorescens* was reported to assist in rot development of onions (Aboaba, 2009).

2.2.2.3 Yeast

Generally, bacteria and fungi have been given the importance in pectinase production as for their function in plant diseases and food processing. However, among 678 identified yeast species (Alimardani-Theuil *et al*., 2011), only some species of genera like *Saccharomyces*, *Rhodotorula*, *Cryptococcus*, *Candida* and *Kluyveromyces* have been described for the pectinase production (Blanco *et al*., 1997). The first publication about yeast endo-polygalacturonase was published by Luh and Phaff (1951) where the report says, *Saccharomyces fragilis*, *Torulopsis*

kefir, *Candida pseudotropicalis* (all later renamed as *Kluyveromyces marxianus*) and *Saccharomyces thermantitonum* (reclassified as *Saccharomyces cerevisiae*) were identified. Pectinolytic yeasts produce polygalacturonase, pectin lyase, pectate lyase and pectin esterase depending on the temperature, pH and substrate availability. For instance, *Kluyveromyces*, *Saccharomyces* and *Candida* produce mainly endopolygalacturonase whereas *Rhodotorula* produces both polygalacturonase and pectin esterase (Alimardani-Theuil *et al*., 2011). *Kluyveromyces wickerhamii* and *Kluyveromyces marxianus* are identified as the major pectinase producers among the tropical yeasts (Da Silva *et al*., 2005). The advantage of yeasts over filamentous fungi in large scale pectinase production is, their growth is relatively simple and the growth medium does not require additional inducers as it does for fungi (Alimardani-Theuil *et al*., 2011).

2.2.3 *Enterobacter aerogenes*

Enterobacter aerogenes is of the phylum of Proteobacteria which is classified into Gamma Proteobacteria. It is a Gram negative motile bacterium. Their optimal temperature ranged is 30 - 37°C. They are mostly aerobic, and also can be classified into facultatively anaerobic bacteria. *Enterobacter aerogenes* found to be in white colour colonies, short rod in shape, colony with smooth margin and convex elevation. This strains is widely distributed in nature such as in freshwater, soil, sewage plants and vegetables, and also in animal and human gastrointestinal tract (Tarawneh *et al*., 2010). It is a nosocomial and opportunistic pathogens, which can cause burns, wounds, urinary tract infections, septicemia and meningitis. Nevertheless they still can produce enzymes. There were researches on lipase production (Kumari *et al*., 2009; Zhang and Guan, 2010), β-lactamases (Tzelepi *et al*., 2000), L-asparaginase (Mukherjee *et al*., 2000) and so on by *Enterobacter*

aerogenes. However, research on pectinase production by *Enterobacter aerogenes* strains has not been patented yet. There was also research conducted on the production of hydrogen from *Enterobacter aerogenes* by using glycerol as the substrate (Markov *et al*., 2011) and evaluation of the ability of this strain to degrade four chlorobenzoic acid compounds in minimal salt medium (Tarawneh *et al*., 2010).

2.3 Industrial applications of pectinase enzyme

Pectinase is one of the upcoming enzymes of the commercial sector. The first commercial application of pectinases was in 1930's. Jayani *et al*., (2005) reported that pectinases have 25% share in the global food enzyme sales. Currently, the production of pectinase conquers about 10% of the overall manufacturing of enzyme preparations (Pedrolli *et al.*, 2009). According to Alimardani-Theuil *et al*., (2011), the world industrial market for pectinases approaches approximately 5% of global enzyme sales. While in the research paper reported by Prathyusha and Suneetha (2011), says that pectinases from food and food Bio products processed waste only, contributes nearly one-third of the world's food enzyme production.

2.3.1 Extraction and clarification of fruit juice

Fruit juices are usually extracted by pressing, but this is not possibly easy with soft fruits like black-currant, strawberries and raspberries (Rombouts and Pilnik, 1980). The crushing of pectin-rich fruits will produce a high viscosity juice which linked to the fruit pulp in a gelatinous structure and interrupts the process of juice extraction by pressing (Pedrolli *et al*., 2009). Pectinase was the first used enzyme in the fruit juice industry mainly for apple juice clarification in the 1930s (Aehle, 2007). Treatment of pectinase in extraction process will degrade the soluble pectin in the pulp and facilitates the pressing and production of juice. When pectinase is boiled, it

is denatured (unfolded) making it harder to connect with the pectin at the active site, and produce more juice. Fruit juice extraction by enzymatic treatment increases yields by more than 90% compared to mechanical extraction method (Pedrolli *et al*., 2009). Besides that, the juices can also be easily filtered and processed. Thus, it decreases filtration time up to 50% (Jayani *et al*., 2005). Freshly-pressed juices have a persistent turbidity which is mainly caused by the presence of pectin. The addition of pectinase results in the removal of cloudy particles and produce clear juice (Kashyap *et al*., 2001).

2.3.2 Oil extraction

The production of oil from emulsion can be improved by enzymes. Pectinases preferably, help for a better separation of oil-water emulsion, where oil is released easily when pectin-protein complexes were hydrolyzed (Kashyap *et al*., 2001). They are useful in the extraction of vegetable oil, coconut germ, palm, sunflower seed, rape seed olived and kernel oils, replacing the traditional method of extraction with organic solvents such as hexane. Citrus oil such as lemon oil also can be extracted by using pectinases (Jayani *et al*., 2005). Furthermore, it is safer and can produce higher yield of oil (Pedrolli *et al*., 2009). A research was conducted by Karim *et al*., (2006) for the extraction of *Moringa oleifera* seed oil using different types of enzymes where Pectinex Ultra SP-L (commercial pectinase) showed 56.5% of oil recovery at optimal acidic condition. In olive oil extraction, the pectinolytic enzymes added during the process of grinding of olives by which the oil will be removed easily in the subsequent separation procedures (Arunachalam and Asha, 2010). In addition to improving the oil yield and stability, enzyme treatment also increases polyphenol and vitamin E content (Sieiro *et al*., 2012).

2.3.3 Degumming and retting of plant fibres

Fibre from ramie is useful in the manufacture of textile materials, however plant gum present on the fibre must be eliminated for its industrial utilization. The traditional method of chemical degumming process consumes a high amount of energy and brings in serious environmental pollutions (Kashyap, *et al*., 2001). Thus, pectinase produced by polysaccharide degrading microorganisms has become the best solution for the process of degumming, maceration, and retting of jute, flax, hemp, ramie and coconut fibres (Pedrolli *et al*., 2009). In addition to being energy conservative, it is also an environmental friendly method of degumming process (Jayani *et al*., 2005). A high pH optimum of pectinase (alkaline pectinase) from microorganisms is reported to be desirable for degumming of plant fibers since a high pH not only prevents contamination but also allows an open fermentation system to be adopted widely (Zheng *et al*., 2001). The scanning electron microscopic studies revealed that a combination of chemical and subsequently polygalacturonase treatment completely removes non-cellulosic gummy material from the surface of ramie bast fibres and sunn hemp bast fibres (Kapoor *et al*., 2001). There was a report that culture supernatant of *Amycolata* sp. which shows high pectate lyase activities was most effective in fibre separation and reduction of gum content in ramie fibre by 30% at 15 hours (Kapoor *et al*., 2001).

2.3.4 Coffee and tea fermentation

In coffee fermentation, pectinase removes the mucilaginous layer of coffee beans which consists of pectic substances (Kashyap *et al*., 2001). Hence, the fermentation stage of coffee processing has been speeded up. Despite that, the cost of drying process also can be reduced. Tea leaves can be macerated completely with

external enzymes that degrade the cell walls compared to the method of maceration by rolling the leaves which only cause partial rupture of the cells. Thus, the use of pectinase enzyme helps to enhance the maceration process which indirectly contributes to the tea fermentation industry (Angayarkanni *et al*., 2002). It has been reported that, the index of tea quality can be increased up to 5% when pectinases is added to cellulases, hemicellulases and proteinases in the tea-leaf fermenting bath (Pedrolli *et al*., 2009). Moreover, the foam forming property of instant tea powder also can be destroyed by pectinase's activity of destroying tea pectins (Arunachalam and Asha, 2010). Fungal pectinase are widely used in the manufacturing of tea.

2.3.5 Wine industry

In the production of red wines, pectinolytic enzymes are added to the macerated fruit before adding the wine yeast, which results in developed visual characteristics (colour and turbidity) compared to untreated wines. Enzymatically treated red wines show an improved chromaticity and stability than the control wines (Jayani *et al*., 2005). The function of pectic enzymes in the winemaking process is also to facilitate the extraction process, increase the juice yield, support filtration and intensify the flavor. Pectic enzymes help a better release of the anthocyanins of the red grapes to increase the colour intensity in the juice, which is later pulled the attention of red wine manufacturers (Kashyap *et al*., 2001). Besides that, pectic enzymes help the breaking down of grapes cell walls and hence to extract the aromatic precursors. The addition of pectic enzymes during extraction and fermentation of grape's musts, increases the susceptibility of aroma precursors to being attacked by beta-glucosidases from the musts, those produced by yeast and bacteria during fermentation or those which are included in commercial enzyme preparations. Thus, it boosts the aroma and flavor of the wines (Sieiro *et al*., 2012).

Pectic enzymes produced by *Aspergillus niger*, *Penicillium notatum*, or *Botrytis cinerea* were proved to be useful in wine making (Kashyap *et al*., 2001).

2.3.6 Textile industry

Textile processing has benefited greatly in both environmental and product quality aspects through the use of the enzymes. Pectinases appear to be the most efficient enzymes for optimal scouring results, being capable of depolymerising the pectin, breaking it down to low-molecular water-soluble oligomers, and thereby improving the absorbency and whiteness of the textile material (Ahlawat *et al*., 2009). Moreover, pectinase also have been used with amylases, lipases, cellulases and hemicellulases to remove sizing agents from cotton, replacing toxic caustic soda used conventionally. In contrast to the traditional method used, pectinase in bioscouring process reduces the usage of toxic alkaline chemicals, safe, eco-friendly and there are no negative side effects on cellulose degradation (Jayani *et al*., 2005). Study done by Solbak and co-workers reported that pectate lyase is the best choice of pectinolytic enzyme in the process of bioscouring (Solbak *et al*., 2005). Another group of researchers had also reported that 80% of pectin were removed from the outer layer of cotton by a purified endo-pectate lyase from *Bacillus pumilus* BK2 (Klug-Santner *et al*., 2006). Wang *et al*., (2007) also said that alkaline pectinase (with lyase activity) produced by *Bacillus subtilis* WSHB04-02 can be used for the bioscouring of cotton knitted fabrics.

2.3.7 Papermaking industry

In papermaking, pectinase is used to depolymerize polymers of galacturonic acids, and later lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching (Jayani *et al*., 2005). Moreover, pectinase treatment does not

damage the strength properties of the pulp (Reid and Ricard, 2000). There was a report on retting of Mitsumata bast by alkaline pectinase from *Bacillus* sp. and *Erwinia carotovora*. These retted basts were used for the preparation of Japanese paper because of the high pulp strength made by bacterial retting (Kashyap *et al*., 2001). Alkaline pectinase produced by *Streptomyces* sp. QG-11-3 combined with xylanase from the same strain was used in bleach-boosting of eucalyptus kraft pulp (Beg *et al*., 2001). On the other hand, report of Sittidilokratna *et al*., (2007) said that the bark which was pretreated with 0.5 and 1.0% of NaOH resulted in the damage of fiber when compared with those treated with pectinase enzyme. Thus, enzyme treated pulp offers high quality fiber and better quality of papers. In addition, the usage of pectinase in paper manufacturing processes allows the reduction of water consumption and thus giving a positive impact to the environment (Alimardani-Theuil *et al*., 2011).

2.3.8 Treatment of pectic industrial wastewater

Naturally, the treatment of wastewater from citrus processing industries containing pectic substances was carried out in few steps; physical de-watering, chemical coagulation, direct activated sludge treatment and chemical hydrolysis which produces methane. The disadvantage side of this method includes, high cost, longer treatment time and brings to environmental pollution because of chemical usages (Arunachalam and Asha, 2010). Thus, the usage of pectinase enzyme from bacteria is an alternate cost effective and environmentally friendly way to selectively remove the pectic substances from the waste water. The pre-treatment of pectic wastewater with alkaline pectinase and alkalophilic pectinolytic microbes help the removal of the pectinaceous material and renders it suitable for decomposition by activated sludge treatment (Jayani *et al*., 2005). In 1987, Tanabe and co-workers had

developed the use of alkalophilic microorganism, *Bacillus* sp. (GIR 621) which produces an extracellular endo-pectate lyase which efficiently remove pectic substances in industrial wastewater treatment (Kashyap *et al*., 2001). Polygalacturonase from *Bacillus* sp. MG-cp-2 can be used in the depectinization of polluting pectic wastewaters from industries, as pectinaceous material can be easily removed by alkalophilic pectinolytic microorganisms (Kapoor *et al*., 2000).

2.4 Production of pectinase by submerged fermentation

Submerged fermentation is a well improved system used in industrial scale to produce a vast variety of microbial metabolites. Submerged fermentation is technically easier than solid state fermentation (Pedrolli *et al*., 2009). Submerged fermentation process conquered the production of commercially important enzymes these days because of the method for sterilization and process control is easy with this system. Large-scale production of microbial enzyme is not possible until after the middle of the 20th century. It is improved fast after the wide usage of submerged fermentation technology which is followed by the development of penicillin fermentations in the 1940's (Waites *et al*., 2001).

2.4.1 Pectinase production in shake flask system

Shake flask is the cheapest and simplest laboratory-scale fermentation method. It is widely used in the study of aerobic fermentations especially to value the production of microorganism, to determine medium composition and quality control of inoculum and raw materials. The shake flask fermentation method does not need continuous control and the handling processes are simple, easy, inexpensive, reduce contamination and they can be operated in parallel. This system needs low cost and energy consumption, easily sterilized and suitable for small scale researches.